High-throughput detection of *Bacillus anthracis* spores using peptide-conjugated nano/micro-beads

Sang Yup Lee

Department of Chemical and Biomolecular Engineering, Center for Ultramicrochemical Process Systems and BioProcess Engineering Research Center, Deparment of BioSystems and Bioinformatics Research Center, Korea Advanced Institute of Science and Technology, Daejeon 305-701, KOREA

- **Goal: Development of a simple microbead-based nanobiosensor for the detection of *Bacillus anthracis* spores**

- **Application: Rapid detection of *B. anthracis* spores**

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14. ABSTRACT
Magnetic bead and Q dots are very attractive materials for detecting B. anthracis spores. Magnetic beads can rapidly isolate B. anthracis spores based on magnetic polarity of the pores, whereas Qdots have ideal characteristics for dense spectral multiplexing, narrow emission range and long lifetime, and can perform simple multiplexed analysis. In this study, they investigated developing a rapid, simple and highly sensitive detection method for detecting B. anthracis spores by using fluorescent nanobeads and magnetic microbeads. They also optimized the conditions for binding affinity of specific capture peptides for spores of two B. anthracis strains including B. anthracis Δ Sterne (pXO1-, pXO2-), and B. anthracis Sterne 34F2 (pXO1+, pXO2-). However, further study needs to be investigated for the optimization of binding characteristics of peptide sequence to B. anthracis spores for sensitive and specific detection. In addition, analysis of the Bacillus spore-coat proteins can be beneficial to fine-tune the detection. The sample preparation for detecting dilute B. anthracis spores in various environmental conditions is also another area to be investigated.

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Background

Bacillus anthracis is a gram-positive, aerobic and spore-forming bacterium that causes anthrax, a lethal disease of human and animals (1-5). It is one of the biggest threats to many countries because of its potential use in bioterrorism, which has actually occurred in the United States of America in the fall of 2001. Once exposed to internal tissues, the spores germinate and vegetate with cell growth, often resulting in the death of the host within several days. Additionally, there is a large overall similarity between *B. anthracis* and other members of the *Bacillus* genus such as *B. cereus*, *B. thuringiensis*, and *B. mycoides*. Therefore, the rapid detection of *B. anthracis* spores in the environment prior to infection is an extremely important goal for human safety. Various biological and chemical techniques have been developed to detect *Bacillus* spores. Among the two most important biological methods are the polymerase chain reaction (PCR) and immunoassays (5-10). PCR, a primer-mediated enzymatic DNA amplification method, requires expensive reagents, molecular fluorophores and considerable effort in sample processing prior to analysis. The detection limit of PCR, which is based on the detection of bacterial *pag*A gene encoding the protective antigen toxic protein, is ~10^3 spores in 3 hours. Immunoassays, which rely on the interaction between antibodies and *B. anthracis* cell surface antigens, can detect 10^5 spores in 15 min. However, in case of immunoassays, specific antibodies should be employed for the desired agents and mobile-phase conditions also should be adjusted depending on their capture, elution, and separation. In addition, although this direct spore detection system is relatively fast, current antibody-based detection method suffers from the lack of accuracy and limited sensitivity, which result in an unacceptably high levels of both false-positive and false-negative responses (8-10). Therefore, a better detection system

needs to be developed.

Magnetic bead is very attractive material because it can be rapidly isolated by magnetic polarity. Qdots have ideal characteristics for dense spectral multiplexing, narrow emission range and long lifetime, and have the potential to simplify the performance of multiplexed analysis (1, 8). In this study, we developed a rapid and simple detection method for *B. anthracis* spores by combining fluorescent nanobeads and magnetic microbeads. It allows detection of specific spores with high sensitivity in less than 1 hour. Also, we optimized the condition allow the best binding affinity of specific capture peptides to spores of two *B. anthracis* strains, *B. anthracis* Δ Sterne ($pXO1^-$, $pXO2^-$) and *B. anthracis* Sterne 34F2 ($pXO1^+$, $pXO2^-$), by using fluorescence confocal microscopy and flow cytometry. Since two species, *B. cereus* and *B. thuringiensis,* are the most similar strains to *B. anthracis* based on genome sequence comparisons, we used them as model strains to be compared before the actual diagnosis of *B. anthracis*.

Materials and methods

Materials. Streptavidin conjugated quantum dot 585 and 525 (≈ 10-15 nm in diameter) were purchased from Qdot (Hayward, CA). Also, streptavidin-conjugated magnetic beads (≈ 1-2 μm in diameter) and portable hand-held magnetic separator were purchased from PureBiotech (San Diego, CA).

Bacterial strains and spores. The *Bacillus* strains used in this study were as follows: the *B. subtilis* DB104 and *B. thuringiensis* 4Q7 were purchased from the Bacillus Genetic Stock Center (BGSC). *B. thuringiensis* HD1 was purchased from the American

Type Culture Collection (ATCC). *B. thuringiensis* KCTC 1510, 1511, 1514, 1516, 1517, 1519 and *B. cereus* 1092 were purchased from the Korean Collection for Type Cultures (KCTC). *B. subtilis* natto was a lab-stock isolated from natto. Expertise with *B. anthracis* ΔSterne ($pXO1^-$, $pXO2^-$) and *B. anthracis* Sterne *34F2* ($pXO1^+$, $pXO2^-$) were carried out at Professor Y.G. Chai's laboratory at HanYang University.

Synthesis and conjugation of capture peptides. The capture peptides were chemically synthesized and purified by high-performance liquid chromatography according to the manufacturer's procedure (Peptron, Daejeon, Korea). At the N-terminus of capture peptides, biotin was attached for conjugation.

Polymerase chain reaction. PCR experiments were performed with a PCR Thermal Cycler (Bio-rad, Hercules, CA) using High Fidelity PCR System. DNA fragments encoding lethal factor 2 were obtained by PCR using the following universal primers and genomic DNA of *B. cereus*, *B. anthracis* ΔSterne and *B. anthracis* Sterne as templates.

Forward primer: 5'-AAGCTTTGAGCAAGTTCATTCAAAAGC-3'

Reverse primer: 5'-ATTGGAAAGTTTTCGGAGCA-3'

Purification of spores. Cells were cultivated in CDSM media at 37℃ or 30℃ with 250 rpm for 48-60 hours (11). Spores mixed with vegetative cells were harvested from 50 mL of the culture by centrifugation (5,000 rpm) and were resuspended in 0.2 mL of 20% (w/v) urografin (Sigma, St. Louis, MO). This suspension was gently layered over 1 mL of 50% (w/v) urografin in a 1.5 mL microcentrifuge tube, and then centrifuged for

10 min at 4℃ and 13,000 rpm. The collected pellet contained only free spores.

Flow cytometric analysis. The purified spores were washed and subsequently resuspended in phosphate-buffered saline (PBS, pH 7.5) solution. Spores were mixed with peptide-Qdot conjugate in PBS buffer and incubated at 30℃ for 1 hour to study binding affinity. Unbound spores and free peptides were removed by washing with PBS solution 3 times. Then, spores were collected by centrifugation at 5,000 rpm at 4℃ for 10 min. Spore-conjugate complexes were resuspended in PBS solution, and fluorescence was measured by fluorescence–activated cell sorting (FACS) analysis with a FACSCalibur instrument and analyzed with CellQuest Pro software (BD Bioscience, Palo Alto, CA).

Image analysis. Fluorescence images were obtained using an LMS 410 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) and Spectrofluorometer (Model VICOPR^3 (PerkinElmer, Shelton, CT). A green and red fluorescence samples were excited by a 488/543 nm HeNe laser, and the images were filtered by a longpass 505/575 nm filter.

Results

To develop a rapid and simple detection of *B. anthracis* spores with capture peptides, five capture peptides were designed based on the previously reported (1) and newly designed sequences as shown in Table 1. Four capture peptides were modified with a biotin-terminal tag for their easy attachment to quantum dots (Qdots) and magnetic

microbeads. Also, one capture peptide was modified with an amine-terminal tag for easy attachment to the COOH-terminated Qdots. During the early-to-mid periods of this project, we used *B. cereus*, *B. thuringiensis*, and *B. subtilis* spores as model strains, because we did not have an access to *B. anthracis* strains in Korea (see below). In order to examine the binding affinities among the peptides and various spores, 5 mg/mL BA1 and BABA peptides were incubated with *B. cereus*, *B. thuringiensis*, and *B. subtilis* spores (ca. 10^9 CFU/mL). Unbound peptides and Qdots were removed by washing with PBS buffer, and spore-peptide-Qdot complexes were analyzed by FACS. We found that BA1 peptide did not bind to *B. subtilis* DB104, *B. subtilis* natto and *B. cereus*, while low-level but detectable binding to the spores of *B. thuringiensis* 4Q7 and *B. thuringiensis* HD1 were observed (Figure 1). These results showed a high degree of specificity in BA1 and BABA binding to *B. thruingiensis* spores, indicating the possibility of binding affinity to *B. anthacis* spores which is very similar to *B. thruingiensis*. For the confirmation of the BA1 peptide specificity, 50 μL of 5 mg/mL BA1 peptides were mixed with Qdot585 and incubated up to 1 hour. After removing unbound Qdots and free peptides by washing with PBS buffer three times, the peptide-Qdot conjugates were incubated with *B. thuringiensis* 4Q7 spores (≈ ca. 2 x 10^7 CFU/mL) for 30 min. Finally, the Qdot-peptide-spore complexes were analyzed by using FACS (Figure 2) and confocal laser scanning microscopy (Figure 3). As shown in Figure 2 and 3, the BA1 peptides bind well to *B. thuringiensis* spores within only 30 min.

At the time of writing the proposal, we thought that it would be impossible to carry out experiments with *B. anthracis* strains due to the security and safety reasons. During the later phase of this project, however, we found that Professor Chai at HanYang

University can carry out *B. anthracis* experiments in his lab. Based on the above results, we applied the strategy developed to detect two *B. anthracis* spores, Δ Sterne ($pXO1^-$, $pXO2^-$) and Sterne ($pXO1^+$, $pXO2^-$), to examine the binding affinities of the capture peptides, BA1, BABA and New to these spores. In brief, Qdot 525 (≈ 4 μL) was incubated with 50 μL of three peptides 5 mg/mL for 1 hour. Subsequently, unbound Qdots and free peptides were removed by washing with PBS buffer three times. Three different peptide-Qdot conjugates were incubated with the three different spores of *B. thuringiensis* 4Q7, *B. anthracis* Sterne and *B. anthracis* Δ Sterne (≈ ca. 2 x 10^7 CFU/mL). *B. cereus* was included as a negative control. Finally, the Qdot-peptide-spore complexes were analyzed by FACS (Figure 4) and confocal fluorescence microscopy (Figure 5, 6 and 7). The successful detection of *B. anthracis* spores with three capture peptides was observed, while low-level fluorescent intensity was detected on *B. thuringiensis* as a negative control. Furthermore, *B. subtilis* DB104, *B. subtilis* natto and *B. cereus* used as other more negative controls did not show fluorescence (data not shown).

We then examined the possibility of detecting *B. antrhacis* spores when they are mixed with other microorganisms as in natural environment. Rapid separation of *B. antrhacis* spores from other spores was carried out using magnetic microbeads. The BABA peptides were incubated with the streptavidin-conjugated magnetic microbeads and washed with PBS buffer to remove unbound peptides. Then, these peptide bound magnetic microbeads were incubated with 1:1 mixture of *B. cereus* and *B. anthracis* Sterne spores. The sample mixture became slightly turbid with brown precipitates after incubation. The precipitate could be moved to the side wall of the tube by using a hand-

held magnet separator. To confirm the separation of two spores (*B. cereus* and *B. anthracis* Sterne), the supernatant (unbound spores) and precipitate (bound spores) fractions were separately spread on Mueller Hinton agar plate for the detection of hemolytic activity. As shown in Figure 8a, the halo-zone caused by hemolytic activity was observed with the unbound spores, but not with the bound spores. Since *B. cereus*, not *B. anthracis*, is supposed to show hemolytic activity, the above result indicates that this method allows simple and rapid isolation of *B. anthracis* spores from *B. cereus* spores. In order to further confirm the successful separation of two spores, PCRs were carried out with universal primers and genomic DNA of each fractions (*B. cereus* and *B. anthracis* after germination) as templates. We found that the PCR products encoding the lethal factor 2 (ca. 400 bp) was only detected in *B. anthracis* Sterne (Figure 8b).

For more convenient high-throughput detection of *B. anthracis* spores, a method of using Spectrofluorometer was developed. BABA peptide was incubated with Qdot 525 and then BABA peptide-Qdot525 conjugates were incubated with *B. anthracis* and *B. thuringiensis* spores. Those complexes were analyzed after incubation (Figure 9a) and profiled by fluorescence intensity assay (Figure 9b). As expected, the *B. anthracis* spores-BABA peptide-Qdot 525 complex was highly fluorescent than it of *B. thuringiensis* due to Qdot 525 emission. Since *B. thuringiensis* also shows weak signals to the peptides, we examined the possibility of negatively screening the system. A negative capture peptide which has binding affinity to only *B. thuringiensis* spores was chemically synthesized. BABA and Negative peptides were incubated with Qdot 525 and Qdot 585, respectively, and were incubated with *B. anthracis* and *B. thuringiensis* spores. Those complexes were analyzed by spectrofluorometer after incubation (Figure 9c). As expected, the *B. anthracis* spores-BABA peptide-Qdot 525 complex was highly

fluorescent due to Qdot 525 emission. Also, the *B. thuringiensis*-negative peptide-Qdot 585 complex was highly fluorescent due to Qdot 585 emission. The weak cross reactions could be distinguished in this manner. These results show that the detection of *B. anthraics* spores, distinguished from *B. thuringiensis* spores, could be successfully detected by using two labeling system employing Qdot 525 and Qdot 585.

In conclusion, methods developed in this studying allow rapid and simple separation and detection of *B. anthracis* spores. However, there are several importants to be made. First, we need optimization of some peptide sequence bound to *B. anthracis* spores for more sensitive and specific detection. Second, analysis of the *Bacillus* spore-coat proteins will be beneficial to fine-turn the interactions, and subsequently detection. Third, a large effort needs to be exerted to sample preparation for the detection of dilute *B. anthracis* spores in various environmental conditions.

Acknowledgments. We thank Professor YG Chai for allowing us to use *Bacillus anthracis* delta Sterne and Sterne strains in his laboratory. This work was supported by the Korean-AFOSR Nanoscience and Technology Initiative from United States Air Force.

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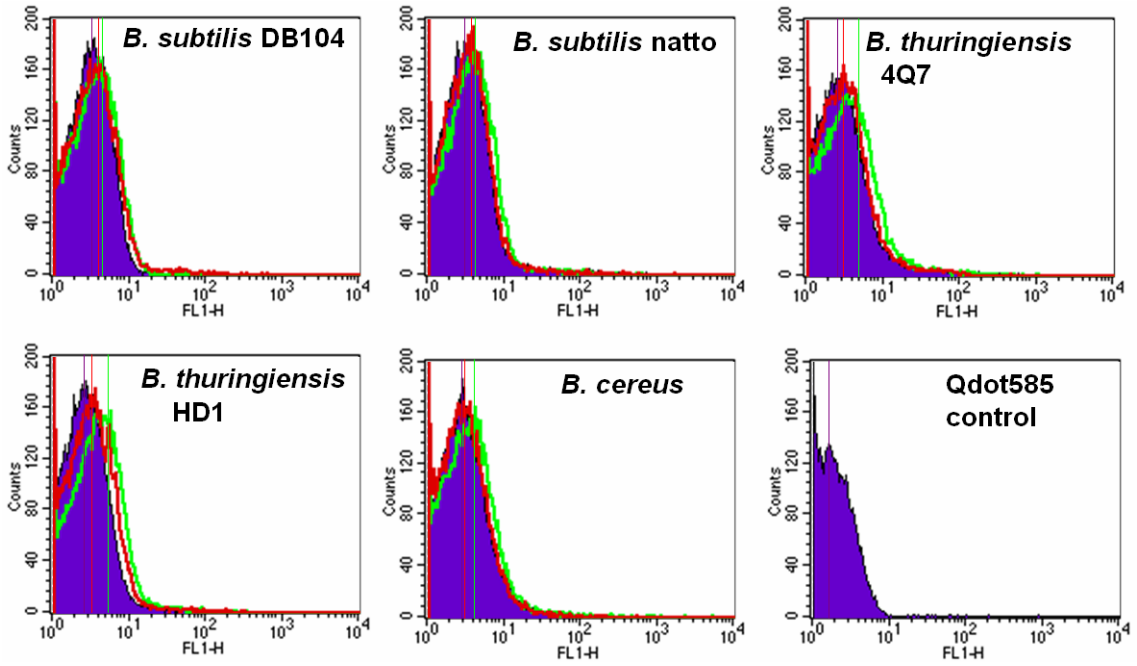

Figure 1. Flow cytometric analysis of various spores bound BA1 peptides. Vertical lines represent mean value of each fluorescence intensity (FL1-H), red line, bound by BA1 peptide; green line, bound by BABA peptide. *B. thuringiensis* spores could be detected, but low-level, by BA1 and BABA peptides.

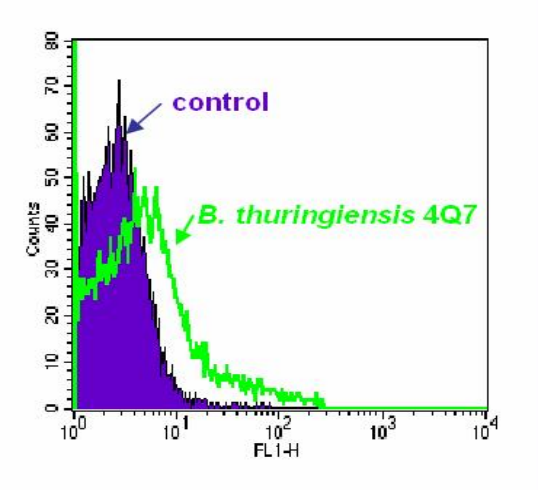

Figure 2. Flow cytometric analysis of *B. thuringiensis* spores bound to Qdot-BA1 oligopeptide complex. The mean fluorescence intensity (*M*) obtained from Qdot-BA1-spore complex (*M*=5.4) was higher than that obtained from negative control (*M*=1.9). After 1 hour incubation of peptide-Qdot conjugates, highly detectable binding to *B. thuringiensis* 4Q7 spores could be observed.

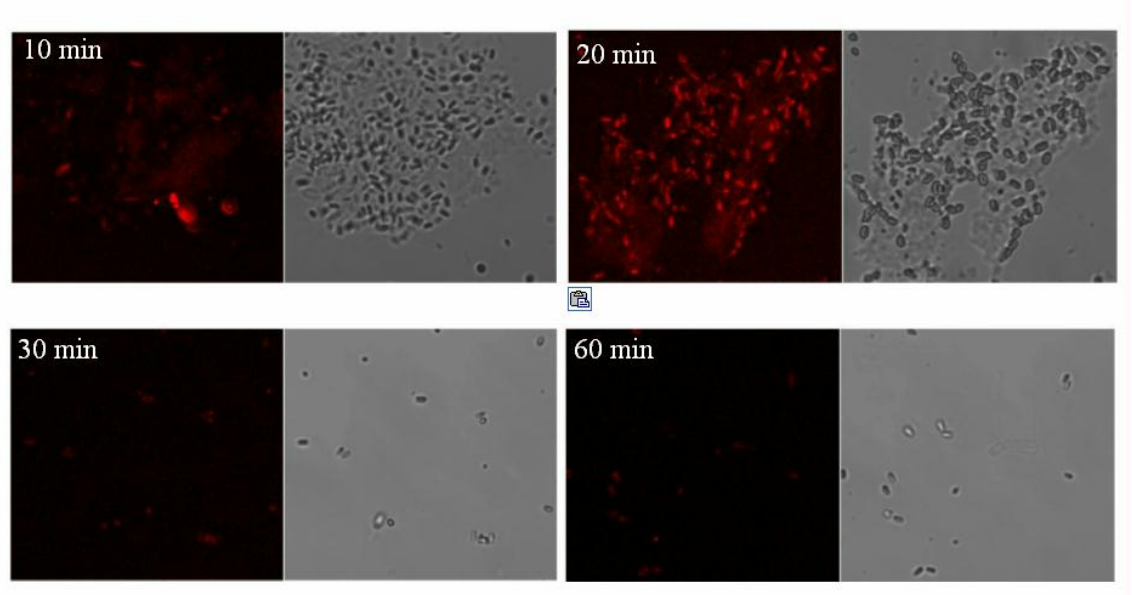

Figure 3. Confocal microscopic images of *B. thuringiensis* spores bound to Qdot-BA1 peptide complex. Specific binging of oligopeptide can be sufficiently observed at even 20 min. BA1 peptide-Qdot conjugates bind well to *B. thuringiensis* spores within 30min.

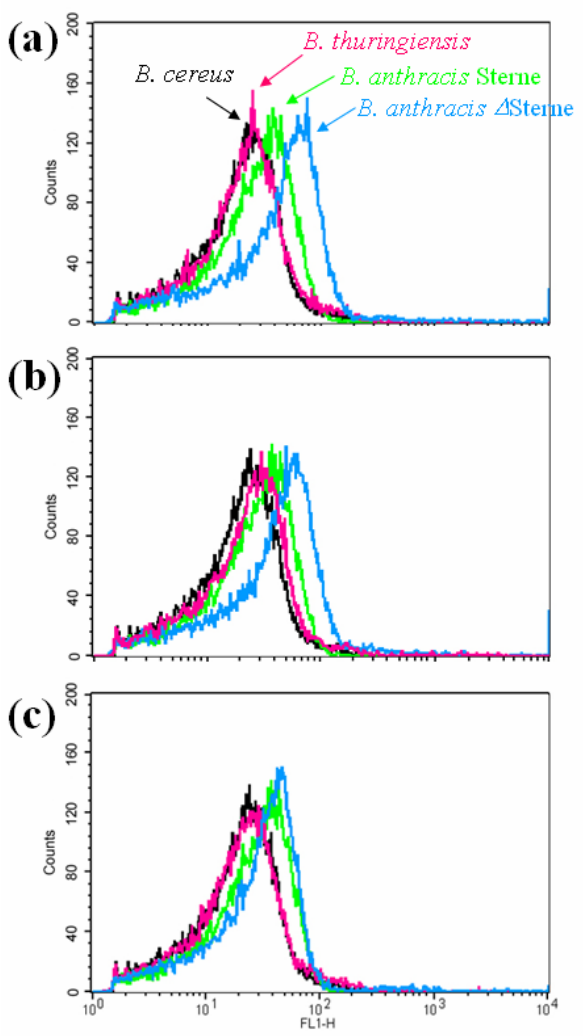

Figure 4. Flow cytometric analysis of various *Bacillus* spores bound to Qdot-peptide complex using (a) BABA, (b) BA1, and (c) New capture peptide. Three different peptide-Qdot conjugates were specifically interacted to *B. anthracis* spores.

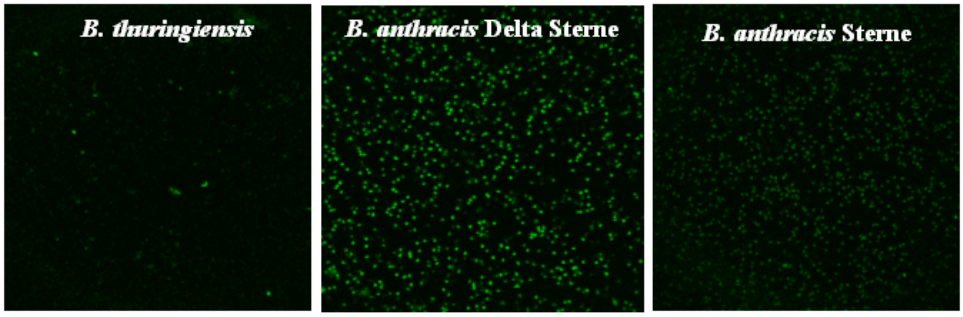

Figure 5. Confocal microscopic analysis of various *Bacillus* spores bound to Qdot-peptide complex using BABA capture peptide. Highly fluorescence intensities were shown in *B. anthracis* strains.

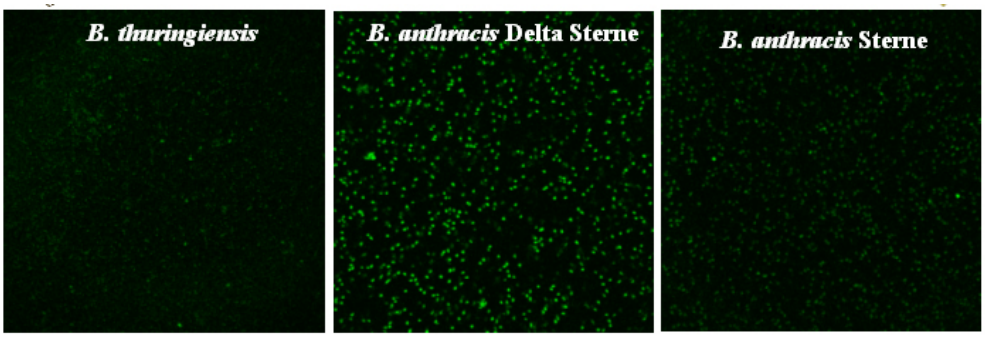

Figure 6. Confocal microscopic analysis of various *Bacillus* spores bound to Qdot-peptide complex using BA1 capture peptide. Highly fluorescence intensities were shown in *B. anthracis* strains.

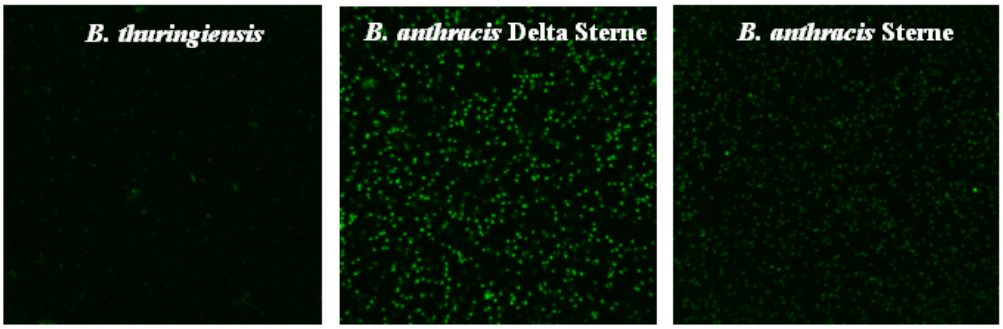

Figure 7. Confocal microscopic analysis of various *Bacillus* spores bound to Qdot-peptide complex using New capture peptide. Highly fluorescence intensities were shown in *B. anthracis* strains.

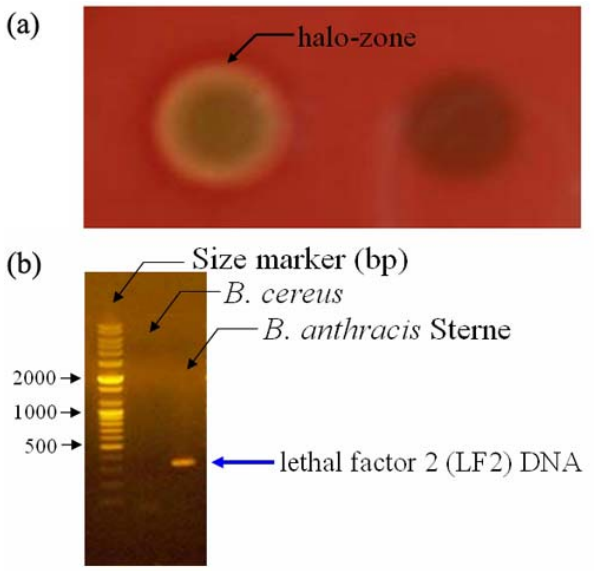

Figure 8. Detection of the bioterroric mediator through magnetic separation. (a) Assay of hemolytic activity. The left is *B. cereus* colony having hemolytic halo-zone, and the right is *B. anthracis* colony. (b) Detection of lethal factor 2 using PCR amplification.

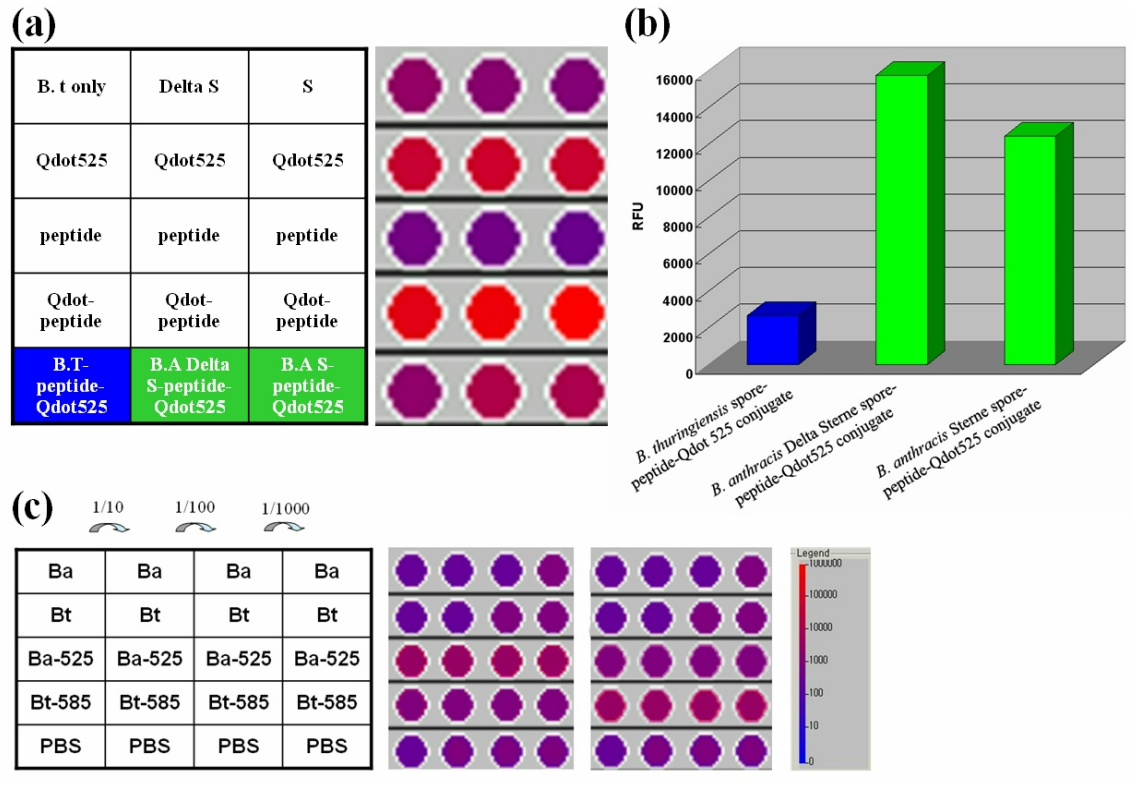

Figure 9. High-throughput detection between *B. thuringiensis* and *B. anthracis* spores. (a) Fluorescence signal using Qdot525-BABA peptide complex. The stronger become Qdot525 signals, the more increase red color. (b) Fluorescence profile using Qdot525-BABA peptide complex. Fluorescence intensity of *B. anthracis* spores compared with *B. thuringiensis* spores was observed an approximately 6-7 fold. RFU, relative fluorescence unit. (c) Fluorescence signal using Qdot525-BABA peptide complex and Qdot585-negative peptide complex. Small amount of samples by serial dilution up to 1000 fold were also detected.

Table 1. Capture peptides used in this study

Capture peptide	Sequence (N-terminus to C-terminus)	Reference
BA-1	biotin-ATYPLPIRGGGC	1
BABA	biotin-ATYPLATYPLC	1
New	biotin-ATYPLATYPLPIRGGGC	1
Negative	biotin-SLLPGLPGGGC	1
Negative-amine	SLLPGLPGGGC	1